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Hyperproduction of Alpha-Toxin by *Staphylococcus aureus* Results in Paradoxically Reduced Virulence in Experimental Endocarditis: a Host Defense Role for Platelet Microbicidal Proteins

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Staphylococcal alpha-toxin targets several cell types which are important components of cardiac vegetations in endocarditis, including platelets, erythrocytes, and endothelial cells. We evaluated the in vivo role of *Staphylococcus aureus* alpha-toxin in experimental endocarditis by using isogenic strains differing in the capacity to produce functional alpha-toxin, including 8325-4 (wild-type strain), DU-1090 (a mutant strain with allelic replacement of the alpha-toxin gene [*hla*]), DU1090(pH35L) (a mutant strain producing a target cell-binding but nonlytic toxin), DU1090(pDU1212) (a variant of DU1090 carrying the cloned *hla* gene on a multicopy plasmid), and DU1090(pCL84::*hla*) (a variant of DU1090 with a single copy of the *hla* gene cloned into the chromosomal lipase locus). In vitro, wild-type alpha-toxin (from parental strain 8325-4) extensively lysed both erythrocytes and platelets. In contrast, mutant alpha-toxin [from strain DU1090(pH35L)] lysed neither cell type. Following exposure to the wild-type alpha-toxin, platelet lysates were found to contain microbicidal activity against *Bacillus subtilis* (but not against *Micrococcus luteus*), as well as against the parental and alpha-toxin variant *S. aureus* strains noted above. Furthermore, lysate microbicidal activity was heat stable, neutralized by polyanionic filters or compounds, and recoverable from anionic filter membranes by hypertonic saline elution. These characteristics are consistent with those of cationic platelet microbicidal proteins (PMPs). Reverse-phase high-pressure liquid chromatography and polyacrylamide gel electrophoresis confirmed the presence of three distinct PMPs (1, 2, and 3) in platelet lysates. In experimental endocarditis, the two variant staphylococcal strains producing either minimal alpha-toxin or nonlytic alpha-toxin in vitro [strains DU1090 and DU1090(pH35L), respectively] exhibited significantly lower virulence in vivo than the parental strain (decreased intravegetation staphylococcal densities). Paradoxically, the two variant staphylococcal strains producing alpha-toxin at supraparental levels in vitro [strains DU1090(p1212) and DU1090(pCL84::*hla*)] also exhibited significantly decreased induction rates and intravegetation staphylococcal densities in experimental endocarditis versus the parental strain. The reduced in vivo virulence of the latter variant staphylococcal strains could not be explained by differences in bacteremic clearance or initial adherence to sterile vegetations (compared to the parental strain). These findings suggest that the reduced virulence exhibited by the variant staphylococcal strains in this model was related to pathogenetic events subsequent to bacterial adherence to the damaged endocardium. Excess intravegetation secretion of alpha-toxin, leading to increased PMP release (secondary to either increased platelet secretion or lysis), may well explain the reduced virulence observed in experimental endocarditis.

Staphylococcal alpha-toxin is an ~34-kDa protein secreted extracellularly by *Staphylococcus aureus* during stationary-phase growth. This protein has been documented as a virulence factor in several focal experimental infections, including keratitis, mastitis, and peritonitis (6, 7, 29). However, the role of alpha-toxin in more systemic staphylococcal infections remains largely undefined. This protein exerts both secretogogue and cytolytic activities against a number of mammalian cell types, including platelets, endothelial cells, and erythrocytes (4, 5, 9, 36, 40, 41), by virtue of target membrane binding, heptamerization, and pore formation (5, 36, 41). Since the aforementioned mammalian targets represent the major cell types present within cardiac vegetations in endocarditis (IE) (20, 38), we hypothesized that staphylococcal alpha-toxin may be an important virulence factor in the pathogenesis of this infection. Thus, the present study was designed to examine the in vivo role of alpha-toxin in the induction and progression of IE by using a well-characterized rabbit model (33) and isogenic *S. aureus* strains with different alpha-toxin secretion profiles. These studies revealed that staphylococcal strains which either are deficient in functional alpha-toxin secretion or hypersecrete functional alpha-toxin exhibit reduced virulence in vivo in experimental IE.

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TABLE 1. Strains used in this study

Strain	Relevant characteristic(s)	Source and/or reference
8325-4	*hla* positive; derivative of ATCC 8325 cured of prophages	
DU1090	Derivative of 8325-4 that is *hla* negative by allelic inactivation	T. J. Foster; 13
DU1090(pDU1212)	DU1090 with *hla* reconstituted on shuttle plasmid; high-level alpha-toxin production	T. J. Foster; 13
DU1090(pH35L)	DU1090 with mutagenized *hla* on shuttle plasmid; production of nonhemolytic alpha-toxin	2
8325-4(pBR322/pCW59)	8325-4 carrying shuttle plasmid without *hla*; chromosomal *hla* expression intact	This study
DU1090(pCL84::*hla*)	DU1090 with cloned *hla* inserted in chromosomal lipase locus by phage insertion (27)	This study
8325-4(pCL84)	8325-4 with phage 11-induced inactivation of chromosomal lipase locus (27)	This study

MATERIALS AND METHODS

Bacterial strains. The strains used in this study are described in Table 1. *S. aureus* 8325-4 is a wild-type, *hla*-positive strain derived from NCTC 8325 that has been cured of prophages and is plasmid free. Strain 8325-4 has been used in previous animal virulence studies (29, 31). DU1090 is a derivative of 8325-4 in which the chromosomal *hla* gene has been inactivated by allelic replacement (kindly supplied by Tim Foster, Dublin, Ireland [31]). Reconstitution of alpha-toxin expression by cloning of *hla* into the *Escherichia coli-S. aureus* multicopy shuttle plasmid pBR322-pCW59 in DU1090 produced DU1090(pDU1212) (31). Similarly, DU1090(pH35L) was generated by introducing a mutagenized *hla* gene on the same shuttle plasmid into DU1090 (29). The mutagenized *hla* gene was created by site-directed mutagenesis of the cloned *hla* gene in *E. coli* to engineer base changes that would replace the histidine at position 35 with leucine. H35L toxin is abundantly produced but is nonhemolytic and non-pore forming, although it retains the ability to bind to target membranes. 8325-4(pBR322-pCW59) is a variant of the parental strain carrying the shuttle plasmid without an *hla* insert (empty plasmid); this strain was created by protoplast transformation (32) of pBR322-pCW59 (*Hin*dIII-*Hin*dIII) into the intermediate *S. aureus* host RN4220 and then transduced into 8325-4 as the final host. Strain DU1090(pCL84::*hla*) is a DU1090 variant in which the *hla* gene has been reconstituted as a single chromosomal copy by inserting the recombinant integration vector into the lipase gene as previously described (27; see below). There were no substantive differences in the 24-h growth curves (in Trypticase soy broth) of any of the staphylococcal strains use in this study, with logarithmic and stationary growth phases achieved in 3 to 4 and 8 to 9 h, respectively (data not shown).

Construction of a single copy of the *hla* gene in mutant DU1090. To avoid the potential problem of increased gene dosage associated with a multicopy shuttle plasmid, we introduced a single copy of the *hla* gene into the chromosome of *hla* mutant DU1090. We excised a 3-kb *Eco*RI-*Hin*dIII fragment from plasmid pDU1212 carrying the *hla* gene. The fragment was then cloned into integration vector pCL84, which inserts preferentially into the lipase gene of the staphylococcal chromosome (27). The recombinant integration vector was introduced by protoplast transformation (18) into recipient strain CYL316 carrying the integrase gene in *trans* (27). The integrants were selected on DM3 agar (18) with tetracycline at 3 μg/ml. Loss of lipase activity in the transformants was confirmed on egg yolk agar (27). The integrated fragment was transduced into *hla*-negative mutant DU1090 with selection for tetracycline resistance and loss of lipase activity. For transduction, phage ϕ11 was used to produce a phage lysate of strain CYL316 containing the mutation. The lysate was then used to infect *hla* mutant DU1090 as previously described (12). Correct integration was verified by Southern blotting with lipase- and *hla*-specific probes as previously described (12). Four transductants (colonies A, B, H, and I) carrying the desired mutation were obtained. Transductant I was chosen for further in vitro and in vivo studies in this investigation; this strain was designated DU1090(pCL84::*hla*).

To verify that inactivation of the lipase gene itself did not influence the virulence of the organism, we constructed a lipase-negative mutant of parental strain 8325-4 by the integration vector method as outlined above, except that pCL84 was used in place of the recombinant integration vector (12, 18, 27). Tetracycline-resistant transformants of recipient strain CYL316 were screened for loss of lipase activity on egg yolk agar. The integrated fragment was then transduced into parental strain 8325-4 to select for lipase-negative transformants as described above (12). Correct integration was verified as described above, by Southern blotting with lipase-specific probes.

Lysis assays. (i) alpha-toxin preparation. The purification of recombinant wild-type alpha-toxin and the mutant H35L toxin has been described previously (29). Briefly, these were purified from the supernatant of 18-h Trypticase soy broth (TSB) cultures of *S. aureus* DU10990(pDU1212) or DU1090(pH35L), respectively. Toxin was purified by using the controlled-pore glass bead chromatography protocol of Cassidy and Harshman (8). Sodium dodecyl sulfate (SDS)-polyacrylamide (12%) gel electrophoresis of 10 μg of protein revealed a single band of approximately 33 kDa corresponding to the monomeric form of each toxin. Wild-type toxin routinely exhibited a hemolytic activity of >20,000 hemolytic units (HU)/mg of protein in a rabbit erythrocyte assay as described by Bernheimer (3). H35L toxin was nonhemolytic at a sensitivity of less than 1 HU/ml in the hemolysis assay. The toxins were stored in aliquots at −70°C until thawed on the day of experimentation.

(ii) Assays for hemolytic activity in culture supernatants. *S. aureus* strains were grown at 37°C in TSB without antibiotic selection for 18 h on an incubator-rotary shaker. After centrifugation at 5,000 × *g* for 10 min to pellet bacteria, the supernatant was diluted and aliquots were added to a 1% suspension of washed rabbit erythrocytes in 0.01 M phosphate-buffered saline (PBS; pH 7.2) containing 0.1% bovine serum albumin in the hemolysis assay described above (3, 24). Data are expressed as mean units of hemolytic activity per milliliter of culture supernatant and represent at least two separate runs.

(iii) Immunoblotting for detection of alpha-toxin. Culture supernatants were prepared as described above, diluted, and applied to a 12% polyacrylamide gel for immunoblot analysis. Antibody generated from the serum of a rabbit immunized with H35L toxin was purified on a protein A-agarose column and used at a dilution of 1:1,000. Immunoblotting was performed as previously reported (29). Anti-H35L antibody detects as little as 5 ng of purified alpha-toxin by this immunoblotting method (29).

The production of alpha-toxin in *S. aureus* is growth phase dependent, with maximal secretion occurring during stationary growth and minimal secretion occurring during logarithmic growth (34). This phasic secretion of alpha-toxin relates directly to the phasic expression of the global regulatory gene complex *agr*, which controls activation of the *hla* gene (12, 25). Therefore, the above-described hemolysis assays and immunoblots of culture supernatants were repeated by using both logarithmic- and stationary-phase preparations.

(iv) Thrombolysis assays. Two distinct techniques were used to quantitate the extent of platelet lysis induced by the wild-type and mutant, nonlytic alpha-toxins. In one assay, washed platelets were suspended in Tyrodes medium and adjusted to an optical density at 600 nm (OD_{600}) of 1.0 spectrophotometrically (~10^9 platelets/ml). Platelets were then exposed to either wild-type or mutant alpha-toxin (100-μg/ml final concentration), and platelet lysis was monitored by measuring the fall in OD_{600} over time. As a positive control, washed platelets were exposed to Triton X-100, a potent mammalian membrane detergent (final concentration, 10% [wt/vol]; Sigma Chemical Co., St. Louis, Mo.). Pilot studies showed that Triton X-100 caused complete lysis of washed platelets within 5 min of exposure. PBS-exposed platelets served as negative controls. The extent of platelet lysis by alpha-toxin was expressed as percent lysis normalized to Triton X-100 (100%). Lactic acid dehydrogenase (LDH) is contained within the cytoplasm of mammalian platelets (26), and its release is an accurate measure of platelet lysis. Thus, in a complementary assay, ~10^9 washed platelets were exposed to either wild-type or mutant alpha-toxin and LDH release was measured over a 30-min coincubation period by a standard enzymatic method (2). Exposure to Triton X-100 (10%) as described above was used as a positive control, and PBS was used as a negative control. LDH release was expressed in terms of micromoles released per minute per 10^9 platelets. The data reported are means of at least two separate experiments.

tPMP. Thrombin-induced platelet microbicidal protein (tPMP) was prepared as previously described, from platelet-rich rabbit plasma (PRP) (44, 45). Platelets were pelleted by centrifugation (1,000 × *g*, 10 min) of the upper two-thirds of the PRP supernatant, and the resulting platelet pellet was washed twice in Tyrode's salts solution (Sigma Chemical Co.) and resuspended in Eagle's minimal essential medium (Irvine Scientific, Santa Ana, Calif.). Preparations rich in tPMP were subsequently produced from washed platelet suspensions (10^8/ml) by stimulation with bovine thrombin (1 U/ml, 37°C, 20 min; Sigma Chemical Co.) in the presence of 0.2 M $CaCl_2$. Residual platelet material was then removed by centrifugation, and the tPMP-rich supernatant was recovered. Substantial purification (approximately 25-fold, ≥80% purity) of tPMP was achieved by anionic adsorption (cellulose acetate-nitrate) and cationic elution as previously described (44). Preparations rich in tPMP were then pooled, dialyzed against 10 volumes of sterile, deionized water (molecular mass cutoff, 3.5 kDa; Spectrapor 3; Spectrum Medical Industries, Los Angeles, Calif.), and stored at −20°C.

Bioactivity of tPMP preparations. The bioactivity of tPMP preparations was determined by previously described methods (44). In brief, bioassays were performed with *Bacillus subtilis* ATCC 6633, a highy tPMP-susceptible indicator organism (44). To determine tPMP bioactivity, *B. subtilis* at 10^4 CFU/ml was added to microtiter wells containing a range of dilutions of the tPMP-rich

preparation to achieve a final concentration of 10^3 CFU per well and a final range of tPMP dilutions ranging from 1:1 to 1:1,024 (final well volume, 200 μl). After 30 min of incubation at 37°C, a 20-μl aliquot was removed from each well, diluted in PBS containing 0.01% (wt/vol) sodium polyanethole sulfonate (SPS; Sigma Chemical Co.) to inhibit further PMP-induced bacterial killing, and quantitatively cultured on 6.6% sheep blood agar. tPMP bioactivity (units per milliliter) was defined as the inverse of the highest tPMP dilution which retained ≥95% lethality versus *B. subtilis* within the 30-min assay period (44). The specific activity of each tPMP preparation was approximately 20 U/μg of protein (~1 μg/ml) as determined by spectrophotometry (λ = 220 nm).

The tPMP susceptibility of the staphylococcal strains used to induce IE in this study was determined by exposing 10^4 bacterial cells to 1 μg of tPMP/ml for 2 h at 37°C in a microtiter well assay system as previously described (44). Three independent runs on separate days were performed. As in prior studies, the breakpoint for in vitro resistance of gram-positive bacteria to tPMP was defined as ≥40% survival after 2 h of exposure (43, 44). *B. subtilis* ATCC 6633 was used as a tPMP-susceptible control. *S. aureus* ISP479R, which exhibits stable tPMP resistance in vitro (17), was used as an additional control for these assays.

Functional characterization of platelet lysates. (i) Bioactivity of platelet lysates. Washed rabbit platelets were prepared from PRP as previously described (44). Following exposure of washed platelets to either purified wild-type alpha-toxin or the nonlytic mutant alpha-toxin isolated from strain DU1090(pH35L) for 30 min at 37°C, residual platelet material was removed by centrifugation and the supernatant (platelet lysate) was recovered. Platelet lysates were then evaluated for bioactivity consistent with that of PMPs. Serial dilutions of the platelet lysates were prepared in Eagle's minimal essential medium and added to *B. subtilis* cells by the same protocol used in the microtiter assay for PMP bioactivity described above. Purified tPMP (45) was used as a positive control. In a parallel microtiter assay, *Micrococcus luteus* was substituted for *B. subtilis* to screen for the presence of platelet lysozyme. *M. luteus* is highly resistant to the microbicidal action of PMPs but is highly susceptible to platelet lysozyme (44). Purified egg white lysozyme (10 μg/ml; Sigma) was used as a positive control.

(ii) Anionic membrane adsorption and elution. As noted above, PMPs are cationic and semipurifiable by anionic affinity techniques (44, 45). Thus, we attempted to adsorb any potential PMPs in platelet lysates with anionic cellulose acetate filters (0.22 μm pore size; Costar, Cambridge, Mass.) (24). Lysates from platelets exposed to either purified wild-type or mutant (nonlytic) alpha-toxin were filtered through siliconized syringe-mounted cellulose acetate filters, and the filtrate was then collected. The filtrate was then serially diluted and tested for PMP bioactivity against *B. subtilis* in the above-described microtiter assay. Following filtration of platelet lysates through cellulose acetate filters, the filters were washed twice with PBS and eluted with 2 ml of 1.5 M NaCl-TN buffer (pH 7.2). Eluates were then dialyzed and serially diluted as described above for the lysate filtrates, and their microbicidal activities against *B. subtilis* were tested as before.

(iii) Charge neutralization of bioactivity in platelet lysates. We have previously shown that the bioactivity of cationic PMPs can be abrogated following admixture with the polyanionic compound sodium polyanethanol sulfonate (SPS) (44). Thus, the microbicidal activities of lysates from platelets exposed to either wild-type or mutant alpha-toxin were tested in the above-described microtiter assay in the presence or absence of SPS (final concentration, 0.01% [wt/vol] in PBS).

(iv) Heat stability. PMPs retain microbicidal activity after being heated to temperatures between 50 and 80°C (44). With the same microtiter assay and *B. subtilis* as the indicator organism, microbicidal activities of lysates from platelets exposed to either wild-type or mutant alpha-toxin were compared before and after 30 min of exposure to 60 or 80°C.

Identification of PMPs in platelet lysates. To identify potential PMPs within platelet lysates, reverse-phase (RP) high-pressure liquid chromatography (HPLC), SDS-PAGE, and acid urea (AU)-PAGE techniques were used as previously described (45). Briefly, following exposure of washed platelets to either wild-type or nonlytic mutant alpha-toxin, lysates were acidified in 30% acetic acid and loaded onto a Bio-Gel P-60 gel filtration column (4.8 by 60 cm; Bio-Rad Laboratories, Hercules, Calif.). The column was developed at room temperature with 30% acetic acid at 30 ml/h, and the eluent fraction protein content was monitored at 280 nm. P-60 gel filtration fractions containing antimicrobial activity against *B. subtilis* in the above-described microtiter assay were pooled, dried by vacuum evaporation (Speed Vac; Savant Instruments, Farmingdale, N.Y.), and dissolved in 5% acetic acid. RP-HPLC was then used as previously described (45) to identify PMPs present within platelet lysates. These identifications were based on elution times by using previously established solvent, gradient, and flow rate conditions (45). The homogeneity of purified peptides within the platelet lysates was then assessed by analytical RP-HPLC. Purified fractions were further analyzed by SDS- and AU-PAGE as previously described (45) to further corroborate RP-HPLC identification of specific PMPs. Gels were stained for 1 to 2 h with 0.1% Coomassie blue R-250 solution containing 5.5% (vol/vol) formaldehyde and then destained for 24 h in water containing 25% (vol/vol) methanol and 0.9% (vol/vol) formaldehyde. As positive controls, known, purified PMPs were run on the same gels; selection of control PMPs was based on the RP-HPLC elution profile of bioactive peaks contained within platelet lysates (45).

Southern blotting. Chromosomal DNAs from *S. aureus* strains were prepared from lysostaphin-lysed cells as described elsewhere (11). DNA was digested with restriction enzymes (New England BioLabs, Beverly, Mass.), transferred onto a Hybond N^+ membrane (Amersham, Arlington Heights, Ill.), and hybridized with gel-purified probes labeled with ^{32}P by the random-primer method (Ready-to-Go Labeling Kit; Pharmacia Fine Chemicals, Piscataway, N.J.). After hybridization, the membrane was washed and autoradiographed as previously described (12).

Isolation of RNA and Northern blot analysis. Overnight cultures of *S. aureus* were diluted 1:100 in CYGP medium (Casamino Acids-yeast extract-glycerol phosphate broth) and grown to the mid-log, late-log and postexponential phases. The cells were pelleted and processed with the FastRNA isolation kit (Bio 101, Inc., Vista, Calif.) in combination with 0.1-mm zirconia-silica beads and a FastPrep reciprocating shaker (Bio 101) as previously described (11). Ten micrograms of RNA of each sample was electrophoresed through a 1.5% agarose–0.66 M formaldehyde gel in MOPS running buffer (20 mM morpholinepropanesulfonic acid [MOPS], 10 mM sodium acetate, 2 mM EDTA, pH 7.0). Blotting of RNA onto Magna Charge membranes (Micron Separations, Inc., Westborough, Mass.) was performed with the Turbo Blotter Alkaline Transfer System (Schleicher & Schuell, Keene, N.H.). For detection of *sar*, *agr*, and *hla* transcripts, the following probes were used: 732-bp *sarA* probe (13, 15), a 513-bp *agrA* probe (nucleotides 3830 to 4342), a 512-bp *hld* probe (nucleotides 999 to 1510) (25), and a 3-kb *hla* probe (31). The probes were radiolabeled with [α-^{32}P]dCTP (Amersham) as described above and hybridized under high-stringency conditions (13). The blots were subsequently autoradiographed.

Rabbit model of IE. The rabbit model of experimental IE was used in these studies as previously detailed (33). Briefly, anesthetized rabbits underwent transcarotid-transaortic valvular catheterization with an indwelling polyethylene catheter to induce sterile valve vegetations. IE was produced by intravenous (i.v.) injection of each of the *S. aureus* strains listed in Table 1 at 24 h postcatheterization. To examine the relative capacities of these strains to induce IE, separate groups of catheterized rabbits were challenged i.v. with a range of inocula of each staphylococcal strain (10^3 to 10^6 CFU) that encompasses the 95% infective doses of most *S. aureus* strains in this model (33, 37). To generate adequate statistical power, at least nine catheterized rabbits were challenged with all of the challenge inocula of each staphylococcal strain.

Assessment of IE. Rabbits were euthanized by rapid i.v. injection of sodium pentobarbital (Abbott Laboratories, Chicago, Ill.) 72 h after infection as described above. At sacrifice, proper catheter position was verified and cardiac vegetations from individual animals were removed, weighed, pooled, homogenized, and then quantitatively cultured as previously described (33). Intravegetation staphylococcal densities were expressed as $\log_{10}$ CFU per gram; culture-negative vegetations were considered to contain ≤2 $\log_{10}$ CFU/g, due to average vegetation weights of ~0.01 g (37). IE was considered as having been induced in any animal with culture-positive vegetations, irrespective of the final vegetation density. Selected vegetation homogenate culture plates were stored at −20°C; bacterial colonies were further analyzed by Southern hybridization for retention of the cloned alpha-toxin (*hla*) gene either in the lipase locus [strain DU1090(pCL84)] or in the multicopy plasmid [strains DU1090(pDU1212) and DU1090(pH35L)] post-in vivo passage (12, 37). In addition, vegetation colonies from animals infected with parental strain 8325-4 carrying the empty shuttle plasmid were analyzed to confirm retention of the plasmid post-animal passage. Moreover, bacterial colonies from these same vegetation homogenate cultures were quantitatively reassessed for retention of prepassage alpha-toxin secretion.

Early adherence of *S. aureus* to vegetations. In vivo adherence assays were performed to compare the abilities of the staphylococcal strains in this study to adhere to sterile cardiac vegetations with the above-described IE model. Separate groups of rabbits underwent aortic catheterization as before and were then challenged 24 h later with ~3×10^7 CFU of the respective *S. aureus* strains. In pilot studies in our laboratory, this inoculum was shown to cause experimental IE in 100% of catheterized rabbits challenged with parental strain 8325-4. For these studies, the vegetation adherence capacity of the parental strain was compared with those of the two strains producing either minimal or nonlytic alpha-toxin [DU1090 and DU1090(pH35L), respectively] and one strain producing excess alpha-toxin [DU1090(p1212)]. At 30 min post-i.v. challenge, catheterized animals were sacrificed and all vegetations from individual animals were removed and quantitatively cultured. The in vivo adherence of each strain was expressed as the mean (± the standard deviation) CFU adhering to sterile vegetations, as previously described (10, 14, 37).

Clearance of bacteremia. It is important to ensure that any differences observed in the ability of the *S. aureus* strains to adhere to valvular vegetations were not related to differences in clearance of bacteremia post-i.v. challenge. Therefore, blood samples were obtained from catheterized rabbits challenged with the various staphylococcal strains at 1 and 5 min post-i.v. inoculation for quantitative culture (10, 14, 37).

Statistical comparisons. Differences in IE induction rate among various staphylococcal strains were compared by the Fisher exact test. Differences in the intravegetation bacterial densities achieved by the various staphylococcal strains were compared by analysis of variance with the Tukey post hoc test for multiple comparisons. P values of ≤0.05 were considered significant.

TABLE 2. Hemolytic activities of purified alpha-toxins and bacterial culture supernatants

Test compound	Mean hemolytic activity[a]
Purified alpha-toxins	
Wild-type toxin	23,250
Cell-binding, nonlytic toxin	<1
Bacterial culture supernatants	
8325-4	400
DU1090	<10
DU1090(pH35L)	<10
DU1090(pDU1212)	1,100
DU1090(pCL84::hla)	650

[a] For purified alpha-toxins, in hemolytic units per milligram. For supernatants, in hemolytic units per milliliter.

RESULTS

Hemolytic activity of alpha-toxins. Purified wild-type alpha-toxin had a mean hemolytic activity of 23,250 HU/mg, while mutant (nonlytic) toxin yielded no detectable hemolytic activity (<10 HU/ml) (Table 2). Early stationary-phase culture supernatants of the parental strain had a mean hemolytic activity of 400 HU/ml, while those of DU1090 (inactivated *hla* gene) and DU1090(pH35L) (producing nonlytic alpha-toxin) had no detectable hemolytic activity. It is noteworthy that the stationary-phase culture supernatants from strain DU1090(pDU1212) (cloned *hla* gene on a multicopy plasmid) had a mean hemolytic activity about three times that of the parental strain (1,100 HU/ml); supernatants from strain DU1090(pCL84::*hla*) (i.e., a single chromosomal copy of *hla*) had a mean hemolytic activity greater than that of the parental strain but less than that of strain DU1090(pDU1212) (650 HU/ml). As expected, none of the logarithmic-phase culture supernatants contained hemolytic activity or alpha-toxin detectable by Western blot analysis. Thus, irrespective of the location of the *hla* gene (normal chromosomal locus, lipase gene integration, or multicopy plasmid), alpha-toxin secretion appeared to be under normal growth phase regulation (12, 25, 34).

Western blot analysis of culture supernatants. As noted in Fig. 1, alpha-toxin was readily detectable in 18-h (stationary-phase) culture supernatants of the parental strain (8325-4), as well as in strains DU1090(p1212) and DU1090(pCL84::*hla*). In addition, alpha-toxin was detected in culture supernatants from strain DU1090(pH35L) containing the site-directed mutant *hla* gene on a multicopy plasmid, encoding a nonlytic toxin. In contrast, no alpha-toxin was detected in culture supernatants of strain DU1090 (inactivated *hla* gene) by Western blot analysis. Moreover, no logarithmic-phase culture supernatant from the above staphylococcal strains exhibited detectable alpha-toxin by immunoblot analysis (data not shown).

Although protein A is a major cell wall constituent and a putative virulence factor in *S. aureus*, some is secreted extracellularly. It is noteworthy that the overexpression of alpha-toxin by the strains with the cloned *hla* gene on either a multicopy plasmid or as a single chromosomal insert did not appear to affect the expression of secreted protein A. As seen in Fig. 1, secreted protein A is readily detected on an immunoblot by its ability to bind to the Fc portion of the same immunoglobulin G (IgG) antibodies utilized to delineate the presence of alpha-toxin in stationary-phase culture supernatants of *S. aureus.*

Thrombolytic activity of alpha-toxins. Triton X-100 caused a rapid decrease in the OD_{600} of the washed platelet suspension

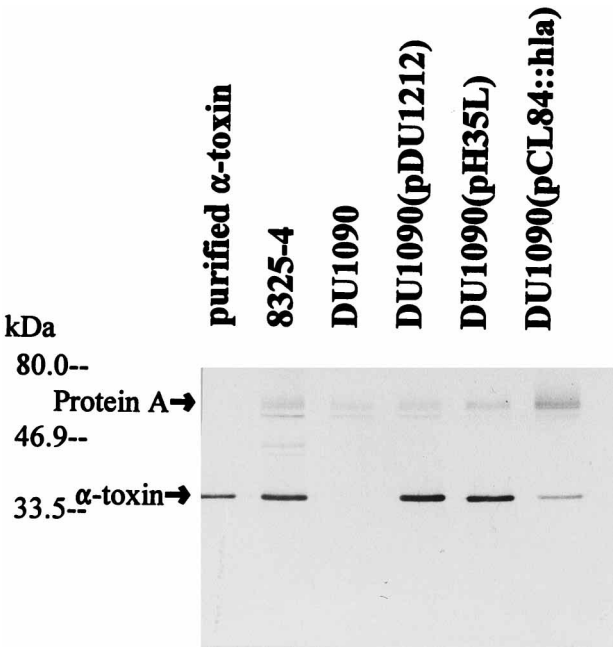

FIG. 1. Western blot for detection of alpha-toxin (34-kDa band) in the culture supernatants of the indicated strains grown to the early stationary phase (18-h cultures). The blot was probed with affinity-purified rabbit anti-alpha-toxin IgG. Lane 1 is the purified alpha-toxin control (50 ng), followed by the culture supernatants of parental strain *S. aureus* 8325-4 and its derivatives DU1090 (lane 3), DU1090(pDU1212) (lane 4), DU1090(pH35L) (lane 5), and DU1090 (pCL84::*hla*) (lane 6). Protein A (which binds to the Fc component of IgG) is detectable as well. The values to the left of the immunoblot are molecular masses of standards (Bio-Rad).

from 1.0 to baseline within 5 min, as measured spectrophotometrically, suggesting complete platelet lysis. Wild-type alpha-toxin resulted in a 76% fall in the OD_{600} of the washed platelet suspension over this same time period, suggesting substantial platelet lysis, while mutant (nonlytic) alpha-toxin caused no detectable fall in the OD_{600}. Triton X-100 and wild-type alpha-toxin resulted in comparable LDH release from washed platelets (7.2 and 6.7 μmol of LDH released per min). In contrast, exposure of washed platelets to alpha-toxin from strain DU1090(pH35L) yielded no detectable LDH release.

***hla*, *sar*, and *agr* gene expression.** Northern blot analysis confirmed that staphylococcal strains DU1090(p1212) and DU1090(pCL84::*hla*) each expressed the *hla* transcript in an amount equivalent to that of strain 8325-4. As predicted, the level of *hla* was maximal during the stationary phase of the growth cycle (Fig. 2). In contrast, *hla* transcription was not detected in mutant strain DU1090 during the entire growth cycle.

To assess the possibility that the *agr* or *sar* locus may be altered in staphylococcal strains with either excess or attenuated alpha-toxin secretion, Northern blots of these strains (parental strain 8325-4, strain DU1090 and strain DU1090 carrying plasmid pDU1212) were performed. Both RNAII and RNAIII of the *agr* locus and *sarA*, *sarB*, and *sarC* of the *sar* locus were transcribed at comparable levels among these strains. Thus, these strains exhibited intact *sar* and *agr* gene expression. It is of importance that these strains demonstrated the normal, growth-phase-related expression of these genes. Thus, during logarithmic growth, *sarA* and *sarB* were maximally expressed and *agr* was minimally expressed. In contrast, during stationary growth, *agr* (RNAII and RNAIII) and *sarC*

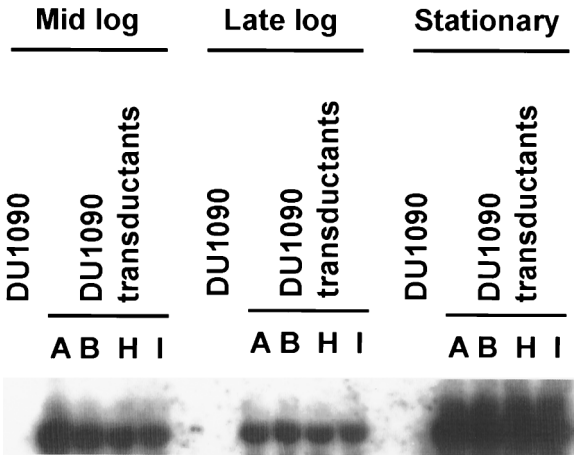

FIG. 2. Northern blot of *hla* transcript in DU1090 (*hla* negative) (lane 1) and DU1090 transductants A, B, H, and I containing single copies of *hla* integrated into the lipase locus of DU1090 (lanes 2 to 5). Ten micrograms of RNA was applied to each lane. The probe was a ^{32}P-labeled 3-kb *Eco*RI-*Hin*dIII fragment of pDU1212. Transductant I [DU1090(pCL84::*hla*)] was selected for use in the in vivo studies.

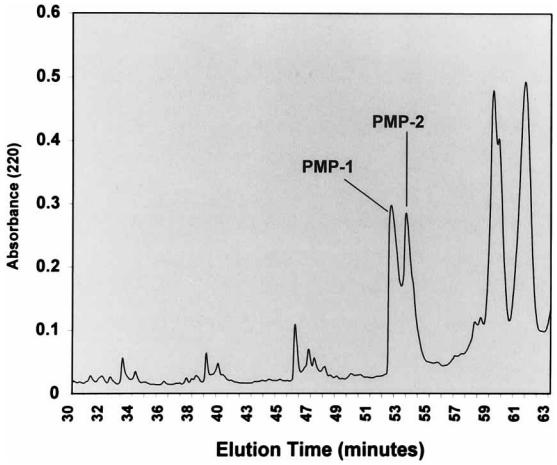

FIG. 3. RP-HPLC chromatogram of lysate from rabbit platelets exposed to alpha-toxin. P-60 fractions containing antimicrobial activity were identified, pooled, lyophilized, acidified in 5% acetic acid, and applied to a C_{18} RP-HPLC column. The column was developed with a linear water-acetonitrile gradient containing 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. Elution was continuously monitored at 220 nm. The major peaks (elution times of 53.0 and 53.7 min) correspond to PMPs 1 and 2 (as previously determined [45]). Note the minor peak at 57.2 min corresponding to PMP 3 (see Fig. 4).

were maximally expressed while *sarA* and *sarB* transcription tapered (data not shown). Thus, in concert with the above data on phasic secretion of alpha-toxin in bacterial culture supernatants, the *hla* gene appeared to be under normal growth phase regulatory control, irrespective of a normal chromosomal or extrachromosomal (plasmid) location (12, 25, 34).

Susceptibility of *S. aureus* strains to PMP. All of the study strains of *S. aureus* used to challenge animals in vivo were susceptible to tPMP, with a range of percent survival at 2 h postexposure of 18 to 32% (24, 26). The tPMP-susceptible control strain (*B. subtilis* ATCC 6633) was completely killed within 30 min of tPMP exposure (44), while the tPMP-resistant control strain (*S. aureus* ISP479R [17]) exhibited >50% survival following 2 h of exposure to tPMP.

Antimicrobial characteristics of platelet lysates. Following exposure of washed rabbit platelets to either purified wild-type or mutant alpha-toxin, platelet lysates were analyzed for microbiological activities in vitro. Lysates from platelets exposed to wild-type alpha-toxin contained ~400 bioactivity U/ml (~2 μg of PMP per ml). This lysate completely killed *B. subtilis* within 30 min of exposure. In addition, all of the staphylococcal strains used to induce IE in this study were susceptible to this platelet lysate (<40% survival after 2 h of exposure in vitro to 200 U/ml (~1 μg/ml). Moreover, after passage of this platelet lysate through anionic membrane filters, the filtrate no longer exhibited microbicidal activity against either *B. subtilis* or any of the study staphylococcal strains in vitro. Exposure of the anionic membrane filters to hypertonic saline yielded an eluate which killed both *B. subtilis* and the study staphylococcal strains. Addition of the polyanionic compound SPS to the platelet lysate also abrogated its microbicidal activities. The microbicidal activity of the platelet lysate was stable following exposure to temperatures between 60 and 80°C for 1 h. Finally, the platelet lysate was inactive against *M. luteus* (an organism killed in vitro by lysozyme [44]). Collectively, these properties are consistent with those of PMPs (44). In contrast, supernatants from platelets exposed to purified alpha-toxin from mutant strain DU1090(pH35L) contained no detectable microbiologic activity against *B. subtilis* or any of the staphylococcal strains used in this study.

Purification of PMPs from platelet lysate. Following exposure of washed platelets to purified wild-type alpha-toxin, AU- and SDS-PAGE of platelet lysates revealed the presence of three cationic, low-molecular-weight peptides compatible with PMPs (45). RP-HPLC revealed the presence of two predominant protein peaks associated with substantial microbicidal activity. (Fig. 3). These peaks eluted at 53 and 53.7 min, consistent with the known elution times of PMPs 1 and 2 (45). The platelet lysate also contained a minor peak at 53.7 min, indicating a minority amount of PMP 3 (purified elution time, 57.3 min; see AU-PAGE gel), which partially coelutes with PMP-2 under such RP-HPLC conditions. To further corroborate the identity of purified peptides, AU-PAGE analysis of elution fractions corresponding to major peaks was performed in parallel with known purified PMPs 1, 2, and 3 (Fig. 4). Collectively, these analyses confirmed that the bioactive peptides derived from platelet lysates were predominantly PMPs 1 and 2, with a minor component consisting of PMP-3 (45).

Experimental IE. Parental strain 8325-4 and strain 8325-4(pBR322/pCW59), carrying the cloning vector without the cloned *hla* gene (empty shuttle plasmid), had equivalent abilities to induce experimental IE across the challenge inoculum range of 10^3 to 10^6 CFU (77 to 100% induction rates versus 75 to 100% induction rates, respectively; data not shown). Mutant strains DU1090 and DU1090(pH35L) containing the inactivated and site-directed mutagenized *hla* gene, respectively, had equivalent capacities to induce IE and were not significantly different from the parental strain (Table 3). In contrast, strain DU1090(pDU1212), containing the cloned *hla* gene on a multicopy plasmid, had significantly lower IE induction rates than the parental strain across a challenge inoculum range of 10^3 to 10^5 CFU. Strain DU1090(pDU1212) also had significantly lower IE induction rates than either strain DU1090 or DU1090(pH35L) with one or more challenge inocula. Strain DU1090(pCL84::*hla*) had substantially lower IE induction rates than parental strain 8325-4 across the challenge inoculum range of 10^3 to 10^6 CFU, although these differences reached statistical significance with the 10^3 CFU challenge inoculum only.

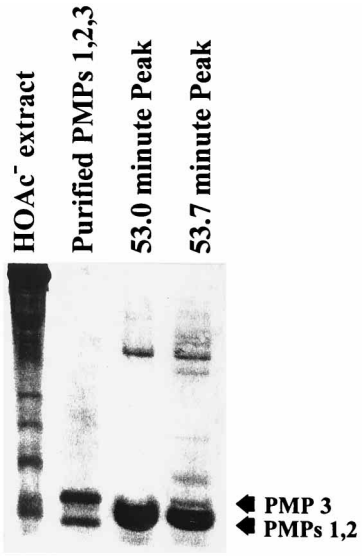

FIG. 4. AU-PAGE of PMPs purified from lysate of platelets exposed to alpha-toxin. This 12.5% acrylamide gel contained 5 M urea buffered with 0.9 M acetic acid, was electrophoresed for 3.5 h at 250 V, and was stained with Coomassie brilliant blue. The first lane contained approximately 100 μg of crude rabbit platelet acetic acid extract ($HOAc^-$ extract). The second lane contained approximately 2.5 μg each of purified PMPs 1 to 3 (PMPs 1 and 2 comigrate under these AU-PAGE conditions). The remaining lanes each contained approximately 10 μg of protein corresponding to peak elution times in the RP-HPLC chromatogram (see Fig. 3). Major peaks from the RP-HPLC purification corresponded to PMP 1 and PMP 2. Note the minor band in the 53.7-min lane immediately above PMP 2. This band likely represents a small amount of PMP 3, which may coelute with PMP 2 in the initial RP-HPLC step (45).

In terms of final mean achievable *S. aureus* densities within cardiac vegetations, no significant differences were observed between the parental strain (8325-4) and its variants carrying either the empty shuttle plasmid (pBR322-pCW59) or the phage-inactivated lipase gene across the challenge inoculum range of 10^3 to 10^6 CFU (3.6 to 9.1 versus 4.0 to 8.5 or 4.4 to 8.2 $\log_{10}$ CFU/g, respectively). In general, the final vegetation densities achieved by each of the other mutant strains were substantially less than that of the parental strain, especially with challenge inocula of 10^4 to 10^6 CFU (Table 4). Thus, mutant strains DU1090 and DU1090(pH35L) achieved significantly lower intravegetation bacterial densities than the parental strain at challenge inocula between 10^4 and 10^6 CFU. It is notable that despite the hyperproduction of alpha-toxin in vitro by mutants DU1090(pDU1212) and DU1090(pCL84::*hla*), both of the latter strains achieved significantly lower intravegetation bacterial densities than did the parental strain. In addition, there were no significant differences in mean vegetation weight in animals infected with the various study strains, supporting the notion that observed differences in mean vegetation density were not related to alpha-toxin-mediated lysis of these lesions.

Bacterial colonies from vegetations of animals were evaluated by Southern hybridization for retention of (i) the cloned *hla* gene on a multicopy plasmid [DU1090(p1212) and DU1090(pH35L)] and (ii) the cloned *hla* gene in the chromosomal lipase locus [DU1090(pCL84::*hla*)]. These studies confirmed retention of the cloned *hla* gene following in vivo passage (data not shown). Similarly, restriction enzyme analysis demonstrated retention of the plasmid *hla* inserts in strains DU1090(pDU1212) and DU1090(pH35L) and confirmed the presence of the shuttle plasmid without an insert in control strain 8325-4(pBR322/pCW59) (data not shown). Furthermore, supernatant hemolytic activities of post-animal passage strains were similar to those obtained from these strains prepassage (data not shown). For example, bacterial colonies from vegetations infected with strain DU1090(p1212) (cloned *hla* gene on a multicopy plasmid) were grown to stationary phase. These culture supernatants contained hemolytic activity equivalent to that of the *S. aureus* strain used to infect catheterized rabbits (~1,100 HU/ml), confirming retention of alpha-toxin secretion postpassage.

Bacteremia clearance and vegetation adherence. The mean bacterial densities in blood at 1 and 5 min post-i.v. challenge in catheterized rabbits challenged with either the parental strain or the variant staphylococcal strains were not significantly different (range, $\log_{10}$ 3.95 ± 0.62 to $\log_{10}$ 4.35 ± 0.14 CFU/ml at 1 min and $\log_{10}$ 1.84 ± 0.57 to $\log_{10}$ 2.33 ± 0.24 CFU/ml at 5 min). Similarly, the early adherence of the parental strain to sterile vegetations in vivo (30 min post-i.v. challenge) was not significantly different from that of the variant staphylcoccal strains (range, 113 ± 49 to 137 ± 51 CFU/vegetation).

DISCUSSION

The pathogenesis of IE is a complex process, involving at least four sequential host-pathogen interactions: (i) endocardial damage, inducing platelet and fibrin deposition at the endocardium (28); (ii) adherence of bloodstream pathogens to damaged endocardial sites (21, 39); (iii) further deposition of platelets and fibrin onto the damaged, infected endocardium (28); and (iv) endocardial reseeding, either hematogenously from distant extracardiac abscesses or locally by intraendocar-

TABLE 3. IE induction rates

Inoculum size (CFU)	No. of animals with IE/no. challenged				
	8325-4 (alpha-toxin$^+$)	DU1090 (alpha-toxin$^-$)	DU1090(pH35L) (alpha-toxin$^+$)	DU1090(pDU1212) (alpha-toxin$^+$)	DU1090(pCL84::*hla*) (alpha-toxin$^+$)
10^3	7/9[a,b]	7/9[a,b]	5/9[c]	3/16[a,c]	2/8[b]
10^4	8/8[d]	6/9[e]	5/9[f]	3/16[d,e,f]	4/7
10^5	7/8[b]	9/9[g]	5/9	4/11[b,g]	6/9
10^6	10/10	9/9	9/9	8/12	5/6

[a] $P < 0.01$ for 8325-4 and DU1090 versus DU1090(p1212).
[b] $P = 0.05$ for 8325-4 and DU1090 versus DU1090(pCL84) and 8325-4 versus DU1090(p1212).
[c] $P = 0.08$ for DU1090(pH35L) versus DU1090(p1212).
[d] $P < 0.005$ for 8325-4 versus DU1090(p1212).
[e] $P < 0.025$ for DU1090 versus DU1090(p1212).
[f] $P = 0.08$ for DU1090(pH35L) versus DU1090(p1212).
[g] $P < 0.005$ for DU1090 versus DU1090(p1212).

TABLE 4. Intravegetation *S. aureus* densities

Inoculum size (CFU)	Mean $\log_{10}$ CFU/g ± SD[a]				
	8325-4	DU1090	DU1090 (pH35L)	DU1090 (pDU1212)	DU1090 (pCL84::*hla*)
10^3	3.6 ± 0.5	4.7 ± 2.1	3.0 ± 2.6	2.55 ± 1.5	2.76 ± 0.61
10^4	8.6 ± 1.8	6.3 ± 2.1	3.0 ± 2.5	2.31 ± 0.9	4.36 ± 2.42
10^5	9.1 ± 0.4	6.6 ± 2.4	3.5 ± 1.5	4.87 ± 2.6	5.15 ± 2.63
10^6	9.1 ± 0.9	7.8 ± 0.7	6.5 ± 3.3	5.39 ± 2.5	6.33 ± 2.8

[a] *P* values: 10^4 CFU of DU1090(pDU1212) or DU1090(pH35L), $P < 0.05$ versus DU1090 and $P < 0.01$ versus 8325-4; 10^4 CFU of DU1090 or DU1090(pCL84::*hla*), $P < 0.05$ versus 8325-4; 10^5 CFU of DU1090(pDU1212) or DU1090(H35L), $P < 0.05$ versus DU1090 and $P < 0.005$ versus 8325-4; 10^5 CFU of DU1090 or DU1090(pCL84::*hla*), $P < 0.05$ versus 8325-4; 10^6 CFU of DU1090(pDU1212); DU1090(H35L), or DU1090(pCL84::*hla*), $P < 0.005$ versus 8325-4; 10^6 CFU of DU1090, $P < 0.05$ versus 8325-4.

dial microorganisms (1, 21, 37, 40). Microbial proliferation within this milieu contributes to the mature, macroscopic vegetation (33). In this paradigm, endothelial cells, erythrocytes, fibrin, and platelets have been traditionally viewed as contributing to the induction and propagation of the vegetative lesion in IE (20, 22, 23, 33, 35). Staphylococcal alpha-toxin is a 34-kDa polypeptide which forms heptameric transmembrane pores in susceptible target cells (36, 41). This toxin has been shown to be an important virulence factor in a variety of experimental *S. aureus* infections, including keratitis, mastitis, and peritonitis (6, 7, 24, 29, 31). Since several of the major components of the vegetative lesion in IE (erythrocytes, platelets, and endothelial cells) are targets for alpha-toxin-induced lethality, the current study was designed to evaluate the potential role of alpha-toxin in this infection by utilizing a well-characterized animal model.

Several important findings emanated from this investigation. The two staphylococcal strains containing either an inactivated *hla* gene or a cloned *hla* gene with a site-directed mutation (yielding a nonlytic toxin) exhibited diminished virulence in experimental IE. The overall IE induction rates of these two strains were not significantly different from those of the parental strain (with or without the empty plasmid). However, the final intravegetation bacterial densities achieved by these two strains were significantly lower than that of the parental strain at several challenge inoculum sizes. Thus, alpha-toxin appears to have a virulence role in IE. Unexpectedly, both staphylococcal strains which secreted alpha-toxin in vitro at suprapaparental levels (cloned *hla* gene either on a multicopy plasmid or in the lipase locus) exhibited reduced virulence in experimental IE. Both the IE induction rates and the final vegetation densities achieved by the latter two strains were significantly less than those of the parental strain at several challenge inoculum sizes.

Induction of bacterial IE is the net result of the ability of a strain to avoid clearance from the bloodstream, to adhere to the damaged endocardium, and to circumvent intravegetation host defenses (16, 46) and proliferate at this site (20–22, 28, 33, 39). In the current investigation, all staphylococcal strains were cleared from the bloodstream at equivalent rates. Moreover, in vivo adherence of the study strains to sterile vegetations was not substantially different. The latter finding is not surprising, since the ability of staphylococci to adhere to vegetations is mediated predominantly by fibrinogen-binding proteins (30). These proteins are controlled principally by the expression of global regulons, such as *sar* and *agr* (12, 25, 30, 34, 42), which remained intact in all strains in the current study. Thus, the diminished virulence in experimental IE of *S. aureus* strains with altered alpha-toxin secretion (diminished, nonlytic, or excessive) is related to postvalvular adherence events.

Following microbial colonization of the damaged endocardium, tissue factor release promotes the local generation of thrombin (18, 19). This protease, in turn, is a potent agonist which prompts the elaboration of antimicrobial peptides from mammalian platelets, termed PMPs (44, 45). PMPs have microbicidal activity against common bloodstream pathogens, including *S. aureus* (43–45). Our laboratory has previously shown that staphylococcal strains which are susceptible to PMPs in vitro are less likely to cause human IE (43) and exhibit diminished virulence in vivo in experimental IE (17). Thus, any events which enhance the intravegetation release of PMPs would be likely to augment the clearance of PMP-susceptible staphylococci. Similarly, any circumstance in which the balance between platelet lysis (via alpha-toxin secretion) and PMP secretion is altered may also modify microbial clearance from infected vegetations. As noted above, variant staphylococcal strains which produced either minimal or nonlytic alpha-toxin were less virulent in experimental IE than the parental strain, especially in final achievable bacterial densities. The nonlytic toxin (H35L) caused neither detectable platelet lysis nor PMP release in vitro. Thus, it is likely that lack of production of alpha-toxin or production of nonlytic toxin would allow an intact population of platelets to be present at the site of microbial colonization, which, in turn, would be subject to normal, thrombin-induced PMP elaboration in vivo. It is of interest that the staphylococcal strain producing the cell-binding but nonlytic alpha-toxin [DU1090(pH35L)] achieved significantly lower bacterial densities within vegetations than did the strain producing no detectable alpha-toxin (DU1090), despite the equivalent capacities of these two strains to bind initially to a damaged endocardium. It is conceivable that the unaltered ability of the mutant alpha-toxin (H35L) to bind to the platelet surface perturbs the capacity of the platelet to serve as a subsequent adhesive surface for reseeding of the vegetative lesion. The latter event is critical to microbial proliferation within the vegetative lesion, with full propagation of the maturing vegetation in IE (37).

Paradoxically, staphylococcal strains which elaborated excess alpha-toxin in vitro exhibited reduced virulence in experimental IE. One hypothesis for this phenomenon is perturbation of a steady-state balance among platelet lysis, PMP release, and microbial killing related to excess alpha-toxin production. Under normal circumstances, platelet lysis and PMP release induced by alpha-toxin might occur only in the immediate proximity of bacterial colonies within the maturing vegetation, killing peripherally located organisms while allowing more centrally located organisms to undergo unrestricted growth. If alpha-toxin hyperproduction develops within the vegetative lesion, excess platelet lysis and PMP production might thus effectively limit microbial proliferation. A second possibility is altered microbial virulence in vivo due to feedback inhibition of *sar* and/or *agr* expression by excess alpha-toxin secretion. Such an event might theoretically suppress the capacity of these organisms to bind to fibrinogen (12, 25, 30, 34) and other matrix proteins at sites of endocardial damage (35, 37), a critical event in the induction and progression of staphylococcal IE (30). A third possibility is altered microbial viruence in vivo due to unbridled alpha-toxin elaboration, leading to an overall suppression of microbial bioenergetics (energy drain via the presence of the multicopy plasmid). The normal expression of *sar* and *agr* genes by alpha-toxin-hyperproducing strains in this study provides compelling evidence against the second possibility. Moreover, the *S. aureus* variant containing the empty multicopy plasmid (i.e., with-

out the cloned *hla* gene) exhibited intact virulence in experimental IE. This observation tends to negate the third possibility (i.e., suppressed microbial bioenergetics leading to altered virulence in vivo). Wild-type (but not nonlytic) alpha-toxin caused extensive platelet lysis in vitro. This event was associated with the release of at least three PMPs (PMPs 1, 2, and 3). It is of note that all of the *S. aureus* strains in this investigation were susceptible to the microbicidal action of PMPs in vitro. Low concentrations of native alpha-toxin can serve as a potent platelet secretagogue, causing nonlytic platelet degranulation in vitro (4, 5). Thus, in the current study, it is not possible to precisely quantitate the contribution of alpha-toxin-induced platelet secretion and lysis to overall PMP release. However, it seems reasonable to hypothesize that excess alpha-toxin secretion within infected vegetations would lead to extensive platelet secretion or lysis, resulting in enhanced release of PMPs and potent local staphylocidal effects.

In summary, it appears that, similar to the in vitro situation (12, 25, 34), alpha-toxin secretion is tightly regulated in vivo. Therefore, within experimental vegetations, pathogenic staphylococci hypothetically elaborate sufficient alpha-toxin to lyse enough platelets to permit proliferation of bacterial cells within the depths of the enlarging vegetative lesions, without releasing excessive quantities of PMPs that might lead to significant bacterial death. Studies are currently in progress to directly assess PMP release within experimental vegetations infected by staphylococcal strains with various levels of alpha-toxin secretion.

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